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GLI1 MODULATION OF THE OUTPUT OF HEDGEHOG SIGNALING IN HUMAN CANCER

Yumei Diao

Karolinska Institutet

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GLI1 Modulation of the Output of Hedgehog Signaling in Human Cancer

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Yumei Diao

Principal Supervisor:
Prof. Peter Zaphiropoulos
Karolinska Institutet
Department of Biosciences and Nutrition

Co-supervisor(s):
Dr. Hui Gao
Karolinska Institutet
Department of Biosciences and Nutrition

Docent John Inge Johnsen
Karolinska University Hospital
Department of Women's and Children's Health

Opponent:
Prof. Sonja Levanat
Ruđer Bošković Institute, Croatia
Division of Molecular Medicine

Examination Board:
Prof. Ann-Kristin Östlund Farrants
Stockholm University
Department of Molecular Biosciences

Prof. Sonia Lain
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Docent Christofer Juhlin
Karolinska University Hospital
Department of Oncology-Pathology

To my parents, brother and sister

Thanks for supporting and encouraging me to move forward

致我的爸爸妈妈和哥哥姐姐

是你们的支持和鼓励让我勇敢前行

Abstract

The Hedgehog (HH) signaling pathway has critical roles in embryonic development and tumorigenesis. Aberrant activation of HH signaling has been known to be involved in several types of malignant tumors. GLI1 is a transcription factor that acts not only as the terminal effector but also represents a pathway target gene, amplifying the HH signal. GLI1 expression/activity is modulated at different levels of gene regulation. The aim of the thesis is to explore the functional alterations in GLI1 transcriptional activity and further investigate the consequences on the output of HH signaling.

Paper I. We discovered an antisense transcript, which is a long non-coding RNA located head-to-head with the gene encoding GLI1, and termed it GLI1 antisense (GLI1AS). The expression of GLI1AS in cancer cells was concordant with GLI1 levels. GLI1AS knockdown upregulated GLI1, increased cellular proliferation and tumor growth in a xenograft model system. GLI1AS overexpression decreased the levels of GLI1, its target genes and cellular proliferation. Additionally, we demonstrated that GLI1 knockdown reduced GLI1AS, while GLI1 overexpression increased GLI1AS, highlighting a regulatory feedback loop on GLI1/GLI1AS expression. GLI1AS acts by modifying the chromatin landscape and reduces the recruitment of RNA polymerase II at the GLI1/GLI1AS locus.

Paper II. We examined the possible interplay of S6K1 and GLI1 signaling in neuroblastoma. mTOR/S6K1 signaling is known to have a role in the development of this disease and recent evidence also implicates the HH pathway. In this paper, it is shown that knockdown of either S6K1 or GLI1 reduces the cellular proliferation of neuroblastoma cells. However, there is little effect of S6K1 on the expression of GLI1 mRNA and protein and on the capacity of GLI1 to activate target genes. No detectable phosphorylation of GLI1 is observed prior or following S6K1 knockdown. Moreover, no additional growth inhibitory effects were detected when PI3K/mTOR and GLI signaling inhibitors were combined. Our results demonstrate that the impact of S6K1 kinase on neuroblastoma cells is not mediated through modulation of GLI1 expression/activity.

Paper III. We investigated HH signaling activation in breast cancer and the contribution of GLI1 to tamoxifen resistance. Our results showed that GLI1 expression was higher in tamoxifen resistant compared to sensitive cells. Tamoxifen resistant cells had stronger ERα transcriptional activity relative to sensitive cells, even though the ERα expression was similar in both cell types. Knockdown of GLI1 attenuated cell proliferation and reduced ERα transcriptional activity in both resistant and sensitive cells, irrespective of estrogen stimulation. Moreover, a positive correlation between GLI1 and ERα expression was identified in breast cancer samples. High GLI1 expression predicted worse distant metastasis-free survival in breast cancer patients. These data suggest that the HH pathway may be a new candidate for therapeutic targeting and prognosis in ERα-positive breast cancer.

Paper IV. We explored the common and differential target genes of GLI1 and edited GLI1, using single molecule RNA sequencing (RNA-seq), via overexpression and siRNA-mediated depletion approaches in rhabdomyosarcoma Rh36 cells. GO analysis revealed that GLI1 and edited GLI1 are involved in developmental and metabolic processes, cellular proliferation, KEGG pathways in cancer, basal cell carcinomas and thyroid cancer. 37 genes were differentially upregulated by edited GLI1, while 29 common target genes, including *FOXS1*, *SOSTDC1* and *SOX18*, were identified by RNA-seq combined with correlation analysis to the FANTOM5 dataset. *SOSTDC1* and *FOXS1* expression was also modulated in HH signaling responsive medulloblastoma Daoy cells by GLI1 knockdown and Smoothened agonist (SAG) treatment. Reciprocally, GLI1 was downregulated in both Rh36 and Daoy cells following FOXS1 knockdown, highlighting a FOXS1/GLI1 regulatory loop.

List of scientific papers

I. Villegas VE, Rahman MF, Fernandez-Barrena MG*, **Diao Y***, Liapi E, Sonkoly E, Ståhle M, Pivarcsi A, Annaratone L, Sapino A, Ramírez Clavijo S, Bürglin TR, Shimokawa T, Ramachandran S, Kapranov P, Fernandez-Zapico ME, Zaphiropoulos PG.
Identification of novel non-coding RNA-based negative feedback regulating the expression of the oncogenic transcription factor GLI1
Mol Oncol. 2014 Jul;8(5):912-26.

II. **Diao Y**, Rahman MF, Villegas VE, Wickström M, Johnsen JI, Zaphiropoulos PG.
The impact of S6K1 kinase on neuroblastoma cell proliferation is independent of GLI1 signaling
BMC Cancer. 2014 Aug 18;14:600.

III. **Diao Y**, Azatyan A, Rahman MF, Zhao C, Zhu J, Dahlman-Wright K, Zaphiropoulos PG.
Blockade of the Hedgehog pathway downregulates estrogen receptor alpha signaling in breast cancer cells
Oncotarget. 2016 DOI: 10.18632/oncotarget.12259.

IV. Rahman MF, **Diao Y**, Vyatkin Y, Azatyan A, St.Laurent G, Kapranov P, Zaphiropoulos PG.
Global analysis of GLI1 and RNA-edited GLI1 target genes
Manuscript

*Both authors contributed equally to the work.

Contents

List of abbreviations

CAM	Chick chorioallantoic membrane
DHH	Desert Hedgehog
EdU	5-ethynyl-2'deoxyuridine
ERE	Estrogen response element
FMCA	Fluorometric microculture cytotoxicity assay
GLI1	Glioma-associated oncogene 1
GLI1AS	GLI1 antisense
GO	Gene Ontology
GSEA	Gene set enrichment analysis
HER2	Human epidermal growth factor receptor 2
HHIP	Hedgehog interacting protein
IHH	Indian Hedgehog
KEGG	Kyoto encyclopedia of genes and genomes
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
PI3K	Phosphoinositide 3-kinase
PSFM	Position specific frequency matrices
PTCH	Patched
RACE	Rapid amplification of cDNA ends
S6K1	Ribosomal p70S6 kinase 1
SAG	Smoothened agonist
SHH	Sonic Hedgehog
SMO	Smoothened
SUFU	Suppressor of Fused
TBP	TATA binding protein
WST-1	Water soluble tetrazolium salt 1

1 Introduction

1.1 Hedgehog signaling pathway

The Hedgehog (HH) signaling pathway was first described in the context of cell fate determination and patterning of *Drosophila melanogaster* [1]. Mutations in the gene encoding the ligand of the pathway resulted in an abnormal spiky-haired phenotype of *Drosophila*, thus the name Hedgehog. Subsequently, it was observed that HH controls cell proliferation, cell differentiation, epithelial-mesenchymal transitions, stem cell maintenance and patterning of tissues and organs during embryogenesis. The three HH homologs, Sonic Hedgehog (SHH), Indian Hedgehog (IHH) and Desert Hedgehog (DHH), are all involved in human developmental processes, with SHH being the most widely studied. The basic HH signaling cascade consists of several steps [2] and the key components of the pathway in *Drosophila* are conserved in human [3].

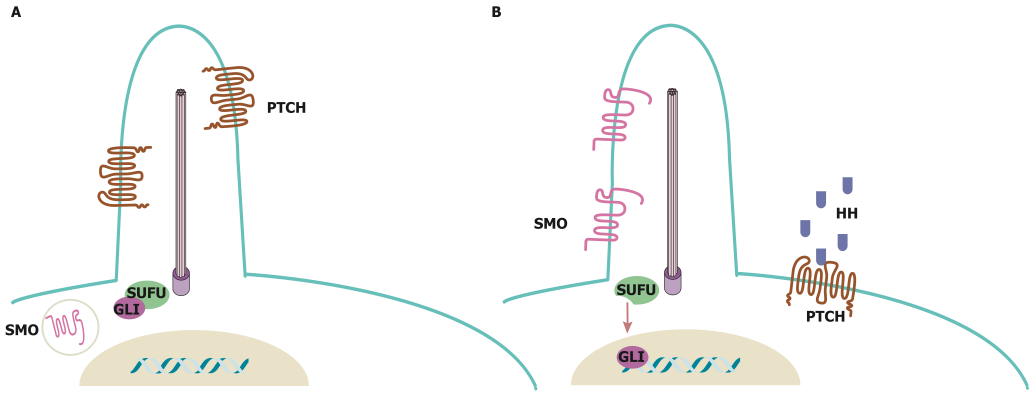

Figure 1. Hedgehog signal transduction.
(A) In the absence of HH ligand, PTCH inhibits the signaling molecule SMO. (B) Secreted HH binds to its receptor PTCH, PTCH inhibition of SMO is relieved and activated SMO releases GLI from cytoplasmic sequestration by SUFU. GLI translocates to the nucleus and upregulates the transcription of target genes.

Primary cilia function as a signaling center for embryonic development and are specialized for HH signal transduction [4]. As shown in **Figure 1**, in the absence of HH, Patched (PTCH1 and PTCH2) inhibits the signaling of the seven trans-membrane domain protein, proto-oncogene Smoothened (SMO). The negative regulator of the pathway, SUFU (Suppressor of Fused), interacts with GLI (Glioma-associated oncogene) in the cytoplasm, preventing GLI mediated transcription. Upon HH secretion, the ligand binds to its receptor PTCH, HH-bound PTCH activates SMO and the activated SMO releases GLI from cytoplasmic sequestration by SUFU [5-8]. In turn, GLI translocates to the nucleus, eliciting the transcriptional activation of target genes. The three members of the GLI family are all important in mediating HH signaling. GLI2 and GLI3 are thought to have both activator and repressor functions, whereas GLI1 only acts as an activator [9, 10]. GLI1 is a terminal signaling effector but also represents a pathway target gene [11], amplifying the HH signal. Its expression levels thus correlate directly with pathway activity. Interestingly, the components of the pathway, PTCH and HHIP (HH interacting protein), are also targets of GLI1.

1.1.1 Aberrant Hedgehog signaling in human cancer

Mutations in HH signaling components and aberrant activation of the HH pathway have been implicated in developmental abnormalities [12, 13] and tumorigenesis [14-17]. It has been reported that numerous types of cancer are associated with abnormal HH pathway activation, including basal cell carcinoma (BCC), medulloblastoma, rhabdomyosarcoma, neuroblastoma [18], breast cancer, meningioma [19, 20], colon cancer [21, 22], and pancreatic cancer [23].

Mutation-driven mechanisms, such as loss of function of PTCH1 and gain of function of SMO, resulting in constitutive activation of the HH pathway, have been demonstrated in BCC [24-26], medulloblastoma [27] and rhabdomyosarcoma [28, 29]. Loss of function mutations in SUFU, the negative regulator of the HH pathway, can also promote the development of BCC [30]. Germline mutations in SUFU increase the risk to develop Gorlin syndrome-associated childhood medulloblastoma [31].

High expression of HH pathway components in breast cancer, including GLI1, is associated with unfavorable prognosis and progressive stages of the disease [32]. It is also shown that the HH pathway has important roles in regulating the development of neural crest stem cells and the sympathetic nervous system [33, 34]. One study found that 58-70% of the tumor samples among 40 human neuroblastoma specimens expressed significant levels of SHH, PTCH1, SMO and GLI2 [35]. Remarkably, this pathway is active in various types of cancer stem cells (CSCs), maintaining their properties, including self renewal and epithelial-mesenchymal transition. Nanog, a stemness gene, is highly expressed in stem cells of the cerebellum and of medullobalstomas, and co-expressed with GLI1. Both GLI1 and GLI2 could regulate the activity of a Nanog reporter construct containing the *cis*-regulatory promoter sequences in mouse and human stem cells [36]. These observations reveal the involvement of the HH pathway in the regulation of stemness properties of cancer cells.

1.1.2 Crosstalk between Hedgehog and other signaling pathways

The signal transduction of the HH pathway can also be mediated through the involvement of additional intracellular signaling cascades [16, 37, 38], including TGF-β (transforming growth factor beta), mTOR/S6K1 (mammalian target of rapamycin/ribosomal p70S6 kinase 1), PI3K (phosphoinositide 3-kinase), MAPK (mitogen-activated protein kinase), HER2 (human epidermal growth factor receptor 2), WNT, NOTCH and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells).

TGF-β-induced GLI2 upregulates GLI1 expression in normal fibroblasts, keratinocytes and various cancer cell lines [39]. Consistently, in the skin of a transgenic mouse model overexpressing TGF-β1, the expression of Gli1 and Gli2 is increased and depends on Smad3, the transcription factor that is activated by TGF-β, with the HH/PTCH/SMO axis not being involved. Similarly, Wang et al [40] showed that mTOR/S6K1 activates GLI1 in a SMO-independent manner. S6K1-mediated phosphorylation of GLI1 increases its transcriptional activity and oncogenic function upon TNF-α induction in esophageal adenocarcinoma.

Moreover, Ramaswamy et al found a crosstalk between HH and PI3K/AKT pathway in breast cancer [41]. They reported that the levels of SMO and GLI1 are significantly elevated in tamoxifen-resistant cells. The PI3K/AKT pathway activates HH signaling, bypassing the blockade of ERα (estrogen receptor alpha) signaling that was elicited by tamoxifen treatment.

The importance and the complexity of HH signaling regulation in various types of cancer not only implicates the HH pathway as a potential pharmacological target but also provides additional possibilities for the development of better therapies based on combinatorial inhibitory approaches. Certainly, it will be a challenge to properly select optimal inhibitors for therapeutic purpose and avoid severe side effects.

1.2 Long non-coding RNA

Advances in sequencing technologies have given researchers the opportunity to more fully appreciate the complexity of the transcriptome. Only 2% of the mammalian genome codes for proteins, while 75% to 95% of the genome is transcribed, producing a large population of non-coding RNAs (ncRNAs) [42], including short ncRNAs (less than 200 nucleotides in length) and long ncRNAs (lnRNAs) (over 200 nucleotides in length) [43, 44]. Similar to protein-coding mRNAs, lncRNAs are mainly transcribed by RNA polymerase II, are 5'capped, polyadenylated and spliced, but have very low coding potential [45]. The promoters of lncRNAs are also marked by active histone modifications, such as H3K4me, H3K4me3, H3K9ac and H3K27ac, but also slightly enriched in markers associated with both repression (H3K27me3) and activation (H3K36me3), compared to those of protein-coding genes [46]. Although most lncRNAs evolve rapidly as far as their sequences and expression levels are concerned, their promoter sequences and their tissue specificities are conserved, similarly to protein-coding genes [47]. A comparison of the characteristics of lncRNA and coding mRNA is shown in **Table 1**.

Table 1. Comparison of lncRNA and mRNA

Characteristics	lncRNA	mRNA
5' capped	✔	✔
3' polyadenylated	✔	✔
RNA poly II transcription	✔	✔
Spliced	✔	✔
Protein coding		✔
No obvious ORF	✔	
Low expression level	✔	
Low sequence conservation	✔	
Fast evolution	✔	

1.2.1 The regulatory mechanisms and biological functions of lncRNA

LncRNAs exert their functions through different mechanisms. LncRNAs can recruit chromatin-modifying complexes and change the landscape of specific DNA loci. LncRNAs can bind and inhibit RNA polymerase II directly, in addition to inhibiting the nuclear translocation of certain transcription factors by binding the specific transporters. LncRNAs undergo conformational changes and fold into specific structures, mimicking DNA-binding sites, and inhibit/enhance the activity of specific transcription factors. LnRNAs can act as scaffolds and recruit proteins into a complex, which is targeted to a specific locus, leading to transcriptional repression or activation. To date, a large number of studies have revealed that lncRNAs regulate embryogenesis, neurogenesis [48], mRNA splicing [49], gene expression [50], control of gene transcription [51], translation [52], gene imprinting [53], disease progression, especially in cancer, including glioma and BCC [54].

1.2.2 Natural antisense transcripts

Global transcriptome analysis has shown that up to 70% of sense transcripts have antisense partners, which are transcribed from the opposite DNA strand, and primarily these are lncRNAs [55]. Antisense transcripts regulate gene expression of the corresponding sense transcripts either in a concordant or discordant way [56, 57]. Knockdown of the antisense may result in the decrease of the sense transcript, which indicates concordant regulation, whereas knockdown of the antisense that increases the sense transcript indicates discordant regulation.

Two classic examples of natural sense/antisense transcript regulation are involved in developmental processes and cancerous growth [55]. (1) *Xist* is a lncRNA, transcribed from the X inactivation centre in the inactive X chromosome, while *Tsix* is transcribed from the opposite strand, overlapping the *Xist* locus [58]. Lee et al reported that both X chromosomes express *Tsix* before X inactivation starts; at the onset of X inactivation, *Tsix* expression becomes monoallelic and persists until *Xist* expression is turned off. They concluded that *Tsix* regulates X inactivation at the early steps, but not at the later, chromosomal silencing steps. (2) *Wrap53*, a natural antisense transcript of *p53*, regulates p53 in a concordant manner [59]. Knockdown of Wrap53 reduces the expression of p53; conversely, Wrap53 overexpression increases the mRNA and protein levels of p53. The underlying mechanism is that a Wrap53-p53 RNA duplex maintains the stability of the p53 mRNA. Interestingly, Wrap53 can also regulate mutant p53. This makes Wrap53 a good therapeutic target for the treatment of cancers with p53 mutations.

The regulatory mechanisms of antisense transcripts can be divided into four types, epigenetic regulation, RNA-DNA interaction, RNA-RNA interaction in the nucleus and RNA-RNA interaction in the cytoplasm [60]. Of these, the antisense RNA-mediated epigenetic modifications have been supported by a large amount of experimental studies. Antisense transcripts can serve as scaffolds for proteins and interact with DNA/chromatin at specific loci, e.g., *cis*-acting epigenetic silencers, Kcnq1ot1 [61] and ANRIL [62]; *cis*-acting

epigenetic activators, Evx1as and Hoxb5/6as [63]; *trans*-acting chromatin remodelers, HOTAIR [64] and HOTTIP [65].

It would be interesting to explore possible antisense transcript(s) against the key components of HH signaling, investigate their regulatory role on the expression of the corresponding sense transcript and their effects in modulating the HH pathway activity. (**Paper I)**

1.3 The role of GLI1 modifications in Hedgehog signaling

It has been reported that GLI1 expression and function can be regulated at different levels, including post-transcriptional and post-translational. Such modulations on GLI1 result in differential functions relative to the wild type GLI1, and influence the activity of the HH pathway.

1.3.1 Edited GLI1

RNA editing is an RNA processing mechanism that includes nucleotide insertion, deletion and modification, but excludes RNA splicing, polyadenylation and degradation [66]. In mammals, the most common type of RNA editing is nucleotide substitution, with A (adenosine) to I (inosine) editing being a prevalent type of RNA editing, which is mediated by the ADAR (adenosine deaminase acting on RNA) enzymes. Since inosine is recognized as a guanosine by most enzymes, A to I editing of protein-coding mRNAs can result in codon changes, which may subsequently induce alterations in protein function [67].

Remarkably, Shimokawa et al reported that the GLI1 mRNA is A to I edited at nucleotide 2179. This A to I substitution causes a codon change from Arginine to Glycine at position 701. GLI1 editing is prevalent in normal human cerebellum, but in medulloblastoma cell lines the extent of this event is severely reduced [68]. Consistently, BCC tumor samples show decreased GLI1 editing in comparison with normal skin. Compared to GLI1, edited-GLI1 exhibits a slightly higher capacity in transcriptional activation and is less sensitive to inhibition by SUFU. However, it is less effective in promoting cell growth. Additional studies revealed novel isoforms of GLI1 generated by alternative splicing [69], including GLI1ΔN (N-terminal deletion variant) [70] and tGLI1 (truncated GLI1) [71], which increase the functional diversity of the GLI1 protein. The structures of the GLI1 mRNA isoforms are shown in **Figure 2**.

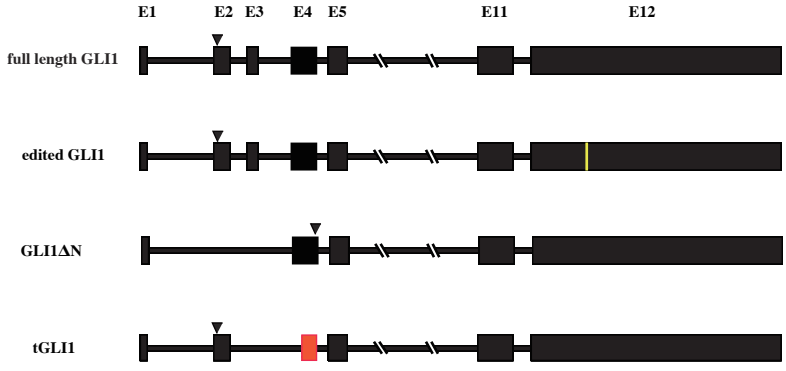

Figure 2. The structures of the different GLI1 mRNA isoforms.
Full length GLI1 mRNA contains 12 exons spanning 3618 nucleotides. Compared to full length GLI1, edited GLI1 has guanosine (inosine), not adenosine, at nucleotide 2179, generated by adenosine deamination, leading to a change from Arginine to Glycine at position 701 in the GLI1 protein, yellow indicates the edited nucleotide; GLI1ΔN mRNA lacks two coding exons, exon 2 and exon 3, resulting in translation initiation from an internal AUG site and a N-terminally truncated protein isoform; tGLI1 lacks exon 3 and part of exon 4, resulting in a shorter protein isoform, red indicates the truncated exon. Exons (E) are numbered and shown by black rectangles. Translation initiation sites are indicated by arrowheads.

Thus, it is worthwhile to explore the differential target genes of GLI1 and edited GLI1 with the help of high throughput RNA sequencing techniques, and to further dissect the consequences and mechanisms underlying HH signaling activation. **(Paper IV)**

1.3.2 Phosphorylated GLI1

Functional diversity in GLI1 can result not only post-transcriptionally, but also from post-translational modifications, including ubiquitination [72], acetylation [73], SUMOylation [74] and phosphorylation [40, 75, 76], with GLI1 phosphorylation being mostly analysed, as shown in **Figure 3** [77, 78].

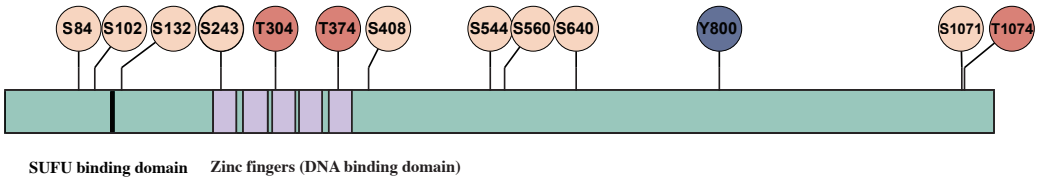

Figure 3. GLI1 protein structure and the phosphorylation sites.
GLI1 contains a SUFU binding domain (black) and five zinc finger domains that bind to DNA (purple). GLI1 phosphorylation sites are shown [78]. Phosphorylation at Serine 243 (S243) and Threonine 304 (T304) has been implicated in increased GLI1 transcriptional activity, while phosphorylation at S102, T374, S408 and T1074 has been linked to decreased GLI1 transcriptional activity. Y, Tyrosine.

Shi et al reported that the member of the Src kinase family, Hck, interacts with GLI1 and phosphorylates GLI1 at multiple tyrosine sites, leading to enhanced transcriptional activity of GLI1 [76]. Additionally, Li et al reported that phosphorylation of GLI1 at Serine 102, Serine 408 and Threonine 1074 by AMPK (AMP-activated protein kinase) reduces GLI1 protein stability [79]. Prevention of AMPK-mediated phosphorylation improved the protein stability and maintained the transcriptional activity of GLI1.

Notably, Wang et al reported that S6K1 phosphorylates GLI1 at Serine 84 independent of SMO, and this increases its transcriptional activity and its oncogenic function in esophageal adenocarcinoma (EAC). The combination of inhibitors to the mTOR/S6K1 and HH pathways had a more potent inhibitory effect on EAC cells than the use of a single agent alone. Taken together, the data suggest that a crosstalk between the mTOR/S6K1 and HH pathways provides a mechanism for SMO-independent GLI1 activation.

Interestingly, both the mTOR and HH pathways are abnormally activated in neuroblastoma, as inhibitors targeting either the mTOR [80] or the HH pathway [81] could block cell growth

in vitro and *in vivo*. Consequently, it was justified to examine the GLI1 phosphorylation status elicited by S6K1 and the possible interplay between these two pathways in neuroblastoma. **(Paper II)**

1.4 Hedgehog pathway and tamoxifen resistance

Based on the expression of molecular biomarkers, ERα, PR (progesterone receptor), and HER2, breast cancer is classified into 6 subtypes, basal-like, HER2-enriched, normal breast-like, luminal A, luminal B and claudin-low, with different subtypes leading to different prognosis [82]. ERα signaling has a critical role in the development and growth of hormone-dependent breast cancer. The domain structure and ligand dependent gene regulation of ERα are shown in **Figure 4**.

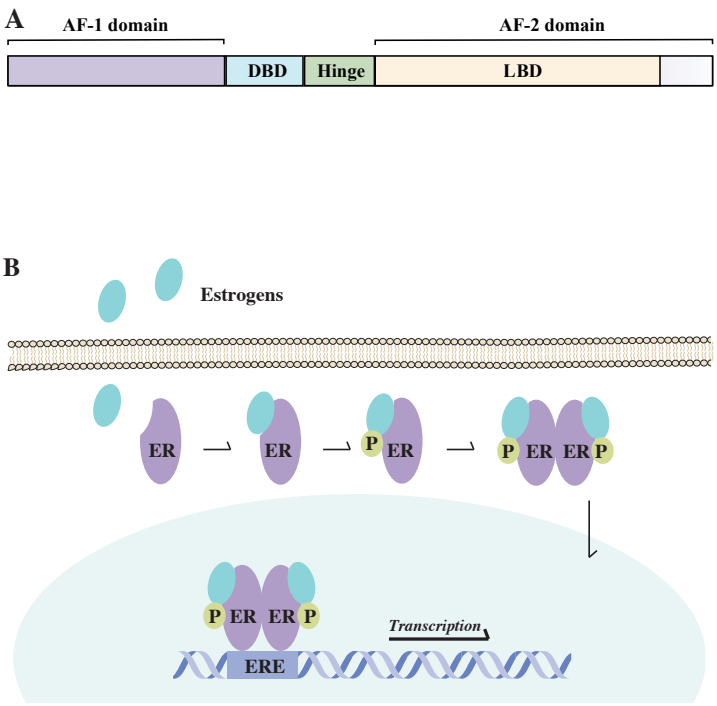

Figure 4. The domain structure of ERα and the classical model of ER mediated gene transcription. (A) ERα contains a number of functional domains, AF-1 domain (activation function 1 domain), DBD (DNA-binding domain), Hinge, LBD (ligand binding domain) and AF-2 domain (activation function 2 domain). The activation of AF-1 domain is ligand-independent, while the activation of AF-2 domain is ligand-dependent. (B) Estrogen binding to the estrogen receptor leads to its dissociation from chaperones, conformational alterations, phosphorylation and dimerization. The ligand-bound ER complex binds to ERE (estrogen response element) in the promoter region and activates the transcription of estrogen-dependent genes via the recruitment of co-regulatory factors.

Tamoxifen is the most commonly used ER modulator for the treatment of ERα positive breast cancer, which accounts for 70% of all human breast cancer cases, including luminal A and luminal B. However in 20-30% of the patients receiving tamoxifen the tumor relapses [83]. This is a major challenge for breast cancer treatment.

1.4.1 The mechanisms of tamoxifen resistance

The mechanisms of intrinsic and acquired resistance to tamoxifen have been extensively investigated [84-87], and may include genetic changes, epigenetic and post-translational modifications. The point mutations in *ERα* E380Q, Y537S and D538G reduce the inhibitory efficacy of tamoxifen [88]. In particular, the Y537S and D538G mutations occur at the N-terminus of Helix 12 in the ligand binding domain (LBD) of ERα, promoting the binding of

constitutive co-activators to ERα and reducing the binding affinity to estradiol and tamoxifen [89]. Additionally, ERα expression may be lost in some patients who develop acquired tamoxifen resistance [90, 91]. Stone et al observed that hypermethylation at *ERα* enhancer regions is associated with a reduction in the DNA binding of ERα in tamoxifen resistant cells, and this downregulates the expression of key modulators of ERα activity. Notably, the methylation status of *ERα* enhancers determines the outcome of tamoxifen treatment [92]. Additionally, ERα phosphorylation is also relevant for the tamoxifen response [93]. For instance, phosphorylation at Serines 102/104/106 [94] and 305 [95] has been linked to tamoxifen resistance.

Moreover, mutations in ERα co-regulators [83, 84], the involvement of non-coding RNAs [96] and cellular kinase signaling pathways, e.g., receptor tyrosine kinases and MAPK pathways [83, 87] are also implicated in tamoxifen resistance.

1.4.2 Hedgehog signaling and breast cancer development

Links between HH signaling activation and breast cancer development have been suggested in a number of studies [97-99]. Hypomethylation of the *SHH* promoter results in an elevated expression of this HH ligand. High expression of SHH and GLI1 indicate high risk of metastasis and poor prognosis in a cohort of 279 patients with invasive ductal breast cancer [100]. Conditional expression of GLI1 in the mouse mammary gland induces the proliferation of epithelial cells and tumor formation [101]. Sun et al showed that estrogen promotes the CSC development and epithelial-mesenchymal transition in ERα-positive breast cancer cells by increasing GLI1 expression [102]. Furthermore, estrogen stimulation increases GLI1 nuclear translocation and regulates the progression from non-invasive to invasive breast cancer [103]. Constitutive activation of HH signaling in *MMTV-SmoM2* transgenic mice caused alterations in mammary gland morphology, increased proliferation, and changed stem/progenitor cell numbers [104]. Taken together, these studies provide insights into the mechanisms of HH signaling activation in the mammary gland and its possible role in breast tumorigenesis.

Interestingly, in 2012 Ramaswamy et al first reported that HH signaling mediates tamoxifen resistance in breast cancer cells [41]. Compared to tamoxifen sensitive cells, tamoxifen resistant cells express higher levels of GLI1 and SMO, and have stronger GLI1 transcriptional activity. In *in vivo* assays, the SMO inhibitor GDC-0449 alone or in combination with tamoxifen suppresses the tumor growth of tamoxifen resistant cells. The proposed mechanism implicates a highly activated PI3K/AKT pathway, which blocks the degradation of GLI1 and SMO in tamoxifen resistant cells. This study suggests that the HH pathway is a potential target for the treatment of tamoxifen resistant breast cancer. This work also sparked our interest to further investigate the role of HH signaling in breast cancer and the underlying molecular mechanisms. **(Paper III)**

2 Aims of the thesis

Aberrant activation of HH signaling has been implicated in many malignancies and includes canonical activation by HH ligands/mutations in components of the pathway or non-canonical activation, which occurs independent of HH/PTCH/SMO via other signaling cascades. The effector of the pathway, the transcription factor GLI1, can undergo diverse modifications at the epigenetic, post-transcriptional and post-translational levels. Consequently, the general aim of the thesis was to investigate the consequences of this GLI1 modulation on the HH signaling activity.

2.1 Dissect the possible regulation of GLI1 by a non-coding GLI1 antisense transcript and uncover the regulatory mechanisms underlying the interplay between GLI1 and GLI1AS.

2.2 Investigate the crosstalk between the mTOR/S6K1 and HH pathways in neuroblastoma cells, examine the GLI1 phosphorylation status and possibly, provide novel therapeutic strategies by targeting both pathways.

2.3 Determine the impact of GLI1 on ERα signaling, and characterize the role of GLI1 in breast cancer and tamoxifen resistance.

2.4 Explore the differential target genes of GLI1 and edited GLI1 by RNA sequencing, and their impact on biological processes mediated by this transcription factor.

3 Results and conclusions

3.1 Paper I

Identification of novel non-coding RNA-based negative feedback regulating the expression of the oncogenic transcription factor GLI1

In silico analysis of EST databases and RACE analysis in rhabdomyosarcoma Rh36, CCA and RMS13 cells revealed that the GLI1AS RNA initiates 155 nucleotides upstream of the major transcription start site of the *GLI1* gene in an opposite orientation, consists of 885 nucleotides and three exons, is 5' capped and 3' polyadenylated but without any long open reading frame (GenBank accession number JX675466).

Expression analysis of GLI1AS showed a remarkable co-regulation with GLI1 in cancer cell lines, basal cell carcinoma and breast tumor samples. GLI1AS transcripts retaining intron 1 or intron 2 were more abundant than transcripts with the introns being removed. The unspliced GLI1AS RNA is preferentially localized in the nucleus, while the spliced GLI1AS RNA transported to the cytoplasm.

SiRNA-mediated GLI1AS depletion in Rh36 cells resulted in an increase of GLI1 expression. On the contrary, GLI1 knockdown decreased GLI1AS levels in both Rh36 and CCA cells. Consistently, EdU incorporation assays showed a decrease in cellular proliferation by GLI1 depletion and an increase by GLI1AS knockdown. The chick chorioallantoic membrane xenograft model demonstrated that treatment of Rh36 cells with GLI1 siRNAs decreased their capacity to form tumors, while an increased tumor weight was observed following treatment with GLI1AS siRNAs.

Expression of the full-length GLI1AS construct but not the 5' or the 3' segment of GLI1AS resulted in a reduction in GLI1 mRNA and protein, and a decrease in *PTCH1* and *PTCH2* expression. These findings suggest that the complete GLI1AS RNA sequence/structure is needed to elicit regulatory effects on GLI1. Interestingly, the expression of *ADAR2*, an unrelated gene on chromosome 21q22.3, was not changed by GLI1AS overexpression, however, the expression of *INHBE*, a gene positioned tail-to-tail to *GLI1AS*, was reduced, albeit not to the same extent as seen for GLI1. Conversely, GLI1 expression in Rh36 cells resulted in an upregulation of GLI1AS, but not INHBE. This suggests that the observed *INHBE* downregulation by GLI1AS overexpression is not an indirect result of the reduced GLI1 levels, but rather an effect of the antisense RNA on the organization of the chromatin locus.

Chromatin immunoprecipitation (ChIP) assays revealed that transcriptional repressive histone markers were increased throughout the *INHBE*/*GLI1AS*/*GLI1* genomic regions following GLI1AS overexpression. The recruitment of RNA polymerase II showed a statistically significant decrease. Thus, GLI1AS appears to act as an epigenetic modifier that represses gene expression at its locus. Furthermore, a concordant upregulation of GLI1 and GLI1AS was elicited by activation of HH or TGF-β signaling.

3.2 Paper II

The impact of S6K1 kinase on neuroblastoma cell proliferation is independent of GLI1 signaling

SiRNA-mediated S6K1 depletion in SK-N-AS neuroblastoma cells reduced cellular proliferation; GLI1 siRNAs treatment also decreased the proliferation but not to the same extent as the S6K1 knockdown, indicating that S6K1 silencing has stronger effects on SK-N-AS cells than GLI1 silencing. The depletion of GLI1, but not S6K1, decreased the expression of SMO, GLI2, GLI3, and PTCH2. Importantly, GLI1 expression was unaffected by knocking down S6K1. Thus, the mechanism of S6K1 on SK-N-AS cell proliferation is not related to the expression of the HH signaling components analyzed.

Western blot and immunoprecipitation analysis indicated that GLI1 expression is not altered by S6K1 knockdown. Moreover, no GLI1 phosphorylation was observed, irrespective of the status of S6K1. Thus, S6K1-dependent phosphorylation of GLI1 is not taking place at detectable levels in SK-N-AS cells.

Ectopic expression of S6K1, the constitutively active mutant S6K1T389E or the function-loss mutant S6K1T389A in SK-N-AS cells could not confer changes in cellular proliferation, neither did GLI1 overexpression. This is in contrast to the observations in esophageal adenocarcinoma cells [40]. Importantly, GLI1 overexpression could not rescue the reduction of cell proliferation elicited by knocking down S6K1. The data suggest that the proliferative effects of endogenous S6K1 and GLI1 have reached saturation in SK-N-AS cells.

The cytotoxicity of the GLI inhibitor GANT61 and the PI3K/mTOR inhibitors AR-12 (OSU03012), CCI-779 and NVP-BEZ235 was evaluated using a fluorometric microculture cytotoxicity assay not only in SK-N-AS but also in SK-N-BE(2) cells. No differences between the log IC_{50} of GANT61 and the log IC_{50} of GANT61 combined with the PI3K/mTOR inhibitors (t-test, $p > 0.05$), except for the combination of GANT61 and CCI-779 in SK-N-BE(2) cells (t-test, $p = 0.032$), were observed.

Thus, we conclude that the impact of S6K1 on the proliferation of the neuroblastoma SK-N-AS cells is not mediated through GLI1 signaling.

3.3 Paper III

Blockade of the Hedgehog pathway downregulates estrogen receptor alpha signaling in breast cancer cells

Expression analysis of key markers of the activity of the HH signaling pathway, i.e., GLI1 and PTCH1, revealed higher expression in the tamoxifen resistant LCC2 breast cancer cells compared to the parental, tamoxifen sensitive MCF7 cells. Notably, the ERα target genes *ADORA1* and *pS2* were upregulated in the resistant cells. Cell viability assays indicated that LCC2 but not MCF7 cells are resistant to 10 μM tamoxifen, however 20 μM tamoxifen kills both cell types. This analysis demonstrates the higher HH signaling activity in the

resistant cells and suggests that ERα activity may also be higher, despite the comparable ERα expression [105].

Depletion of ERα resulted in a major reduction of the cell proliferation in both cell lines, highlighting their dependence on ERα. Depletion of GLI1 also reduced the cell proliferation of the two cell lines, but to a lesser extent. These observations are in-line with the significance of ERα in breast cancer cells [106] and indicate that GLI1 can modulate proliferation not only in tamoxifen resistant but also in tamoxifen sensitive cells.

Estrogen response element (ERE) luciferase reporter assays showed that GLI1 depletion reduces ERα activity both in MCF7 and LCC2 cells, irrespective of the presence of estrogen. Importantly, the basal level of the ERα transcriptional activity was higher in LCC2 compared to MCF7 cells, an observation in-line with the expression pattern of the ERα target genes *ADORA1* and *pS2*. These findings suggest an interplay of GLI1 and ERα signaling in both tamoxifen resistant and sensitive cells.

GLI1 depletion decreased the expression of *PTCH1, ERα* and its target genes, *IL20*, *ADORA1* and *pS2*, in the context of estrogen treatment, while limited effects were observed without addition of estrogen. The results were confirmed using two additional ERα-positive breast cancer cell lines, ZR751 and T47D. Western blot analysis demonstrated that GLI1 depletion downregulated ERα in both MCF7 and LCC2 cells. Consistently, ChIP analysis revealed decreased ERα binding at the promoter region of its target gene *pS2* following GLI1 depletion in the presence of estrogen.

GANT61 treatment resulted in a dose-dependent reduction of the viability of MCF7 and LCC2 cells. GANT61 co-administration with tamoxifen further decreased the cell growth inhibition elicited by tamoxifen. GLI1 depletion also enhanced the impact of tamoxifen in reducing the proliferation of the two cell lines. Similar enhancement of the tamoxifen impact by GLI1 depletion was observed in ZR751 and T47D cells. Thus, the role of GLI1 for the proliferation of ERα-positive breast cancer cells may be exploited for therapeutic purposes, and drug targeting of GLI1 could enhance the tamoxifen efficacy in the treatment of breast cancer.

Gene expression analysis revealed that the expression of *GLI1* positively correlates with *ERα* gene *(ESR1)* and the ERα targets genes *pS2* and *GREB1* using a dataset of breast cancer samples from 286 individuals [107]. High GLI1 expression was also found to be associated with poor distant metastasis-free survival (DMFS) in 126 patients with Grade 1, ERα-positive breast cancer in the Kaplan-Meier Plotter dataset [108]. These findings suggest that GLI1 may represent not only a therapeutic target, but could also be a valuable prognostic marker for breast cancer patients.

3.4 Paper IV

Global analysis of GLI1 and RNA-edited GLI1 target genes

RNA from rhabdomyosarcoma Rh36 cells transfected with GLI1, edited GLI1 [68] or GLI1 siRNAs were used for single molecule RNA sequencing (RNA-seq) via the Helicos platform. Upregulated genes with a Z score over 2 were initially selected in the GLI1 or edited GLI1 overexpression; downregulated genes with a fold difference over 1.2 were also selected in the GLI1 depletion. Combining the overexpression/depletion datasets allowed the identification 477 GLI1 and 480 edited GLI1 target genes, 197 of which were common. These common target genes were used for GO analysis and curated gene-set enrichment analysis.

Moreover, in a parallel approach the 1000 genes with the highest Z score in the GLI1 and edited GLI1 overexpression datasets were subjected to a Spearman correlation with the gene expression FANTOM5 datasets of 833 tissues. In both cases a correlation higher than by chance was revealed. Combining the GLI1 and edited GLI1 datasets increased the Spearman correlation. Inclusion of the criterion of downregulation following GLI1 depletion further increased the correlation, resulting in a list of 29 genes. Furthermore, increasing the stringency to a two-fold downregulation following GLI1 depletion reduced the list to 8 genes (one of which being the known GLI1 target *PTCH1*). Interestingly, 25 out of the 29 selected genes were present in the previous list of the 197 common targets. In addition, heat maps generated for the 29 selected genes, using both the GLI1 overexpression and knockdown datasets, showed clear up- and downregulation respectively, compared with 29 randomly selected genes. Finally, a Spearman correlation distribution analysis demonstrated a higher correlation with GLI1 expression of the 29 selected genes in comparison to the 29 random genes.

Among the 29 selected target genes, 14 genes were validated by RT-qPCR in Rh36 cells following GLI1 depletion. Moreover, two genes, *FOXS1* and *SOSTDC1*, were also validated in GLI1 and edited GLI1 overexpressions in Rh36 cells and in medulloblastoma Daoy cells following Smoothened agonist (SAG) treatment. Notably, the induction of *SOSTDC1* and *FOXS1* by SAG was suppressed in CRISPR/Cas9-mediated *GLI1* knockout Daoy subclones. Surprisingly, FOXS1 knockdown resulted in the reduction of *GLI1* expression, indicating a FOXS1-GLI1 regulatory loop.

To identify target genes that are differentially upregulated by GLI1 and edited GLI1, the top 1000 genes with the highest Z score in the GLI1 or edited GLI1 overexpressions were filtered for absence in the reciprocal overexpression and for downregulation in GLI1 depletion, followed by a Spearman correlation with the gene expression FANTOM5 datasets. The correlation of the selected list for GLI1 overexpression was in the random range, while a list of 37 genes with a statistically significant correlation was generated for the edited GLI1 overexpression, three of which were also validated by RT-qPCR in biological replicates. Interestingly, expression of the typical GLI1 target gene *HHIP*, but not *PTCH1*, was also found to be preferentially upregulated by the edited compared to the non-edited GLI1 in Rh36 cells.

4 Future perspectives

Hedgehog-GLI1 signaling is widely studied using a variety of different methodological approaches, but no reliable Western blot/ChIP graded antibodies against GLI1 are available, possibly due to the complex GLI1 structure, and this narrows the effectiveness of GLI1 functional studies. To further expand on some of the projects in this thesis, we will first aim to add an epitope tag on the C-terminus of endogenous GLI1 using the CRISPR/Cas9 technology. Secondly, we will employ the adeno-associated virus (AAV) expression system for tagged GLI1/edited GLI1 overexpression [109], in order to overcome both the lack of effective antibodies and the low efficiency of transient plasmid transfection.

In Paper III, we showed that GLI1 depletion downregulated ERα expression, reduced ERα transcriptional activity and its recruitment on the *pS2* promoter, suggesting that GLI1 has a role in modulating the transcription of ERα/ERα target genes. By modulating GLI1 expression levels through CRISPR/Cas9 and/or AAV systems, we may detect GLI1-ERα interactions by immunoprecipitation and clarify the involvement of GLI1 in ERα regulated gene expression using GLI1 ChIP-qPCR or ChIP-seq. To investigate the possibility of GLI1 acting as a co-regulator in ER transcriptional activation, we could do ChIP and re-ChIP using antibodies against ERα and the epitope tag, respectively.

In Paper IV, we performed CRISPR/Cas9 mediated *GLI1* knockout in Daoy cells and obtained three Daoy subclones with compromised GLI1 activity and HHIP expression upon SAG treatment. Next, we will verify the genomic changes in the *GLI1* gene by genomic PCR and reverse transcription PCR. We will continue to validate the differential targets of GLI1 and edited GLI1 by RT-qPCR. To confirm the GLI1 target potential of *FOXS1* and *SOSTDC1*, we will engage into luciferase reporter assays, using promoter constructs of these two genes combined with GLI1 overexpression. We will proceed with GLI1/edited GLI1 ChIP-qPCR to validate the identified target genes, including *FOXS1* and *SOSTDC1,* as direct targets in Rh36 cells using the AAV expression system. To further examine the role of FOXS1, we will determine the impact of siRNA-mediated FOXS1 depletion in Daoy cell proliferation with or without SAG treatment, followed by expression analysis of key components in HH signaling.

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